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DN 105:168806
TI Directed selection of differentiation mutants of Streptomyces noursei using chemostat cultivation
AU Noack, D.
CS Forschungsbereich Biowiss. Med., Dtsch. Akad. Wiss., Jena, DDR-6900, Ger. Dem. Rep.
SO J. Basic Microbiol. (1986), 26(4), 231-9

FUNDAMENTAL STUDIES OF URINARY TRACT INFECTION WITH SERRATIA INTERACTION BETWEEN BACTERIA OF DIFFERENT GENERA AND SPREADING OF R PLASMID TO SERRATIA.
AU MASU C
CS DEP. UROL., HIROSHIMA UNIV. SCH. MED.
SO MED J HIROSHIMA UNIV, (1986) 34 (4), 453-472.
CODEN: HDIZAB. ISSN: 0018-2087.
FS BA; OLD
LA Japanese

Adaptive reversion of a frameshift mutation in Escherichia coli
AU Cairns, John; Foster, Patricia L.
CS Dep. Cancer Biol., Harvard Sch. Public Health, Boston, MA, 02115, USA
SO Genetics (1991), 128(4), 695-701
CODEN: GENTAE; ISSN: 0016-6731

TI Modification in penicillin-binding proteins during in vivo development of genetic competence of Haemophilus influenzae is associated with a rapid change in the physiological state of cells
AU Dargis, M.; Gourde, P.; Beauchamp, D.; Foiry, B.; Jacques, M.; Malouin, F.
CS Cent. Rech., Cent. Hosp., Ste-Foy, PQ, G1V 4G2, Can.
SO Infect. Immun. (1992), 60(10), 4024-31

I Decreased susceptibilities to teicoplanin and vancomycin among coagulase-negative methicillin-resistant clinical isolates of staphylococci
AU Sieradzki, Krzysztof; Villari, Paolo; Tomasz, Alexander
CS The Rockefeller University, New York, NY, 10021, USA
SO Antimicrobial Agents and Chemotherapy (1998), 42(1), 100-107

Triclosan and antibiotic resistance in Staphylococcus aureus
AU Suller, M. T. E.; Russell, A. D.
CS Pharmaceutical Microbiology, Welsh School of Pharmacy, Cardiff University, Cardiff, CF10 3XF, UK
SO Journal of Antimicrobial Chemotherapy (2000), 46(1), 11-18
CODEN: JACHDX; ISSN: 0305-7453

TI Augmentation of antibiotic resistance in Salmonella typhimurium DT104 following exposure to penicillin derivatives
AU Carlson, S. A.; Ferris, K. E.
CS National Animal Disease Center, Pre-harvest Food Safety and Enteric Disease Research Unit, Agricultural Research Service, USDA, Ames, IA, USA
SO Veterinary Microbiology (2000), 73(1), 25-35

TI Augmentation of antibiotic resistance in Salmonella typhimurium DT104 following exposure to penicillin derivatives

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Modification in Penicillin-Binding Proteins during In Vivo Development of Genetic Competence of *Haemophilus influenzae* Is Associated with a Rapid Change in the Physiological State of Cells

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By using a whole-cell labeling assay with ^{125}I-penicillin V, we observed a reduction in the binding of the radiolabeled β-lactam to four or five penicillin-binding proteins (PBPs) in *Haemophilus influenzae* cells cultivated under specific conditions. PBPs 3A, 3B, 4, and 6 were altered after the growth of bacteria in diffusion chambers implanted in the peritoneal cavity of rats. PBP 2 was also modified when cells were cultivated in human cerebrospinal fluids. Because this observation may have important consequences on the efficacy of β-lactams during antibiotic therapy, we characterized the physiological state of bacteria cultivated in animals in the hope of explaining how such important changes in cell properties develop in vivo. Since the development of natural genetic competence occurs at the stationary phase of growth in *H. influenzae*, we used a DNA transformation assay to evaluate the physiological state of bacteria grown in diffusion chambers implanted in rats. Chromosomal DNA isolated from an antibiotic-resistant donor strain was mixed with bacteria in diffusion chambers. At different times during a 5-h incubation period, recipient bacteria were collected from the chambers, CFU were determined by plate counting, and antibiotic-resistant transformants were isolated on selective plates. Genetic competence rapidly developed in cells grown in rats, and the frequency of transformation by test DNA was elevated. Electron microscopy revealed an irregular cell shape and blebs at the surface of bacteria cultivated in animals and in cerebrospinal fluids. In an attempt to induce a similar physiological state in vitro, we supplemented broth cultures with cyclic AMP or synchronized cultures by a nutritional upshift. No changes in PBPs were observed with supplemental cyclic AMP or during a single cell cycle. Finally, a reduction in the affinity of PBPs for ^{125}I-penicillin V identical to that observed in bacteria grown in rats was observed in cells isolated from the stationary phase of growth in vitro. These results clearly indicate that *H. influenzae* cells grown in animals undergo a rapid change to a physiological state similar to that found in late-stationary-phase cultures in vitro. This observation indicates that the rational design of future and improved antibiotic therapy of *H. influenzae* infections should consider cell properties of slow-growing or latent bacteria.

Haemophilus influenzae is an important human pathogen causing severe infections in young children and immunocompromised adults (16, 32). Newer β-lactams are some of the currently recommended antibiotics for the treatment of these infections (6, 14). β-Lactams produce their initial bacteriostatic effect through the inhibition of their cellular targets, the penicillin-binding proteins (PBPs), which are essential enzymes involved in the cell wall peptidoglycan synthesis of bacteria (47). In general, the susceptibility of gram-negative bacteria to β-lactam drugs results from the relative effects of the outer membrane permeability and the periplasmic β-lactamase on the periplasmic β-lactam concentration. In turn, the periplasmic β-lactam concentration needed to affect the inner membrane-bound PBP targets depends on the affinity of these PBPs for the β-lactam (33). In some cases, because of this interplay of factors, bacterial resistance may develop because of a slight decrease in the affinity of the β-lactam for the PBP targets (23).

The enzymatic activities of PBPs and cell wall composition and conformation are directly affected by the physiological state of bacteria, which in turns depends on the growth conditions (42, 43). We designed this study to allow the characterization of the physiological state of *H. influenzae* cells grown in an environment that simulates the in vivo growth conditions occurring during infections and possibly to reveal specific cell properties that may influence the inhibitory activity of β-lactam antibiotics.

In the present study, we used rat intraperitoneal diffusion chambers (25) to allow the efficient recovery of animal-grown bacteria. Since the development of natural genetic competence occurs at the stationary phase of growth in *H. influenzae* (40), we used a DNA transformation assay to evaluate the physiological state of bacteria and monitored changes in the PBPs of *H. influenzae* cells cultivated in vivo and in human cerebrospinal fluids to determine how rapidly these growth environments can affect the cellular targets of β-lactam antibiotics.

MATERIALS AND METHODS

Bacterial strain and in vitro growth conditions. The medium used for the growth of *H. influenzae* Rd was brain heart infusion (Difco Laboratories, Detroit, Mich.) supplemented (sBHI) with 15 μg each of hemin (Eastman Kodak Co., Rochester, N.Y.) and β-NAD (Sigma Chemical Co., St. Louis, Mo.) per ml. Rat peritoneal fluids collected from sterile diffusion chambers (see below) and pooled human

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TABLE 1. Biochemical analysis of human cerebrospinal and rat peritoneal fluids

Fluid	Concn (mM except where indicated) of:							
	Glucose	Total protein (g/liter)	Calcium	Chloride	Magnesium	Phosphorus	Potassium	Sodium
Human cerebrospinal[a]	3.95 (2.2–3.7)	0.27 (0.2–1.5)	1.29 (1.20)	132 (130)	1.20 (1.0)	0.49 (0.50)	2.85 (2.8)	154.5 (140)
Rat peritoneal								
Noninfected	7.7	5	0.96	125	0.31	1.23	2.9	145
Infected[b]	4.3	10	0.24	106	0.03	1.22	4.0	138

[a] Numbers in parentheses represent the approximate values observed in healthy humans (49).
[b] Rat peritoneal fluid was collected from diffusion chambers that contained bacteria for an incubation period of 24 h.

cerebrospinal fluids (kindly provided by L. Côté and G. Chassé, Laboratoire et Service d'Infectiologie, Centre Hospitalier de l'Université Laval, Ste-Foy, Québec, Canada) were also used to cultivate *H. influenzae* in test tubes. The compositions of these biological fluids is given in Table 1. M-IV medium (11) was used as a nongrowth medium prior to cell synchronization. Strain Rd was maintained on chocolate agar plates (Quélab Laboratories, Montreal, Quebec, Canada).

Diffusion chambers and in vivo growth conditions. Diffusion chambers (15 by 10 mm) were constructed from polypropylene tubing (Nalgene Co., Rochester, N.Y.) as described before (25). Millipore 0.22-μm-pore-size MF filters (Millipore Corp., Bedford, Mass.) were cut to the diameter of the chambers and attached by melting the chambers to affix the filters. The chambers were autoclaved. Overnight cultures were diluted 1:10 in sBHI and allowed to reach exponential growth (A_{600}, 0.08, 0.4, or 0.8). The cells were washed twice in an equal volume of saline and injected through a needle hole melted through the side of the chambers. The hole was subsequently closed by use of a hot glass rod. The chamber volume was approximately 500 μl. Sprague-Dawley female rats weighing approximately 250 g were anesthetized with ketamine HCl (87 mg/kg) and xylazine (13 mg/kg) given intraperitoneally. Four chambers were placed in the peritoneal cavity through a small longitudinal incision in the abdomen. Animals were sacrificed with carbon dioxide before the chambers were removed. Cells were removed from the chambers and collected for various tests.

Labeling of PBPs. The procedure for binding radiolabeled penicillin to whole bacterial cells was previously described (26). Radiolabeling of PBPs was performed by a modification of the method of Spratt (39) as described by Preston et al. (36) with ^{125}I-penicillin V as the labeled β-lactam (2). *p*-(Trimethylstannyl)penicillin V was kindly provided by Larry C. Blaszczak (Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, Ind.) and was iodinated with Na^{125}I (Amersham Canada Ltd., Oakville, Ontario, Canada). Cells in phosphate-buffered saline were incubated with 20 μg of ^{125}I-penicillin V (37.3 Ci/mmol) per ml for 30 min at room temperature. The labeled cells were washed and loaded for electrophoresis on discontinuous 0.1% sodium dodecyl sulfate–10% polyacrylamide gels (20). Dried gels were exposed to Kodak X-Omat film for autoradiography at −20°C for 7 days. The relative reduction in binding of the radiolabeled penicillin to a particular PBP was determined by scanning the PBP profiles obtained on the X-ray film with a Bio-Rad model 620 video densitometer.

DNA transformation assays. Chromosomal DNA from β-lactamase-negative, moxalactam-resistant *H. influenzae* T-1,3 (34) was extracted by the procedure of Silhavy et al. (38) and used to transform competent *H. influenzae* Rd cells to the resistance phenotype as described before (27). For the in vivo transformation assay, an overnight culture was diluted 1:10 in sBHI and allowed to reach exponential growth (A_{600}, 0.4). The cells were washed twice in an equal volume of saline and mixed with chromosomal DNA (1 μg of DNA per 0.5 ml of cells in saline), and the mixtures were placed in diffusion chambers for implantation in rats. At different times during the incubation of bacteria in rats, diffusion chambers were collected, CFU were determined by plate counting, and antibiotic-resistant transformants were isolated on selective plates containing 0.25 μg of moxalactam per ml of medium.

Synchronization of cell growth. Cells from an overnight culture were washed twice in M-IV medium and adjusted to an A_{600} of 0.2. The cells were incubated at 37°C for 45 min, and synchronization of cell growth was achieved by a nutritional upshift: the cell suspension was diluted 1:4 in prewarmed sBHI. Growth was monitored by recording the optical density of the culture in a Beckman DU-64 spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.) and by viable cell plate counting at intervals after synchronization. Whole-cell labeling of PBPs was also performed at each time point.

Effect of cAMP on the expression of PBPs. Overnight cultures were diluted 1:10 in sBHI and allowed to reach exponential growth (A_{600}, 0.4). The cultures were washed twice in an equal volume of sBHI and supplemented with 0.1, 1, 10, and 100 mM cyclic AMP (cAMP) (Sigma). The cultures were incubated at 37°C for 0 to 6 h, and samples were collected at various times during growth for the examination of PBPs.

Electron microscopy. The procedure used for the preparation of samples for electron microscopy was described before (1, 10). After withdrawal from diffusion chambers or growth in culture medium (sBHI or M-IV), bacterial cells were collected by centrifugation and suspended in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) for 24 h at 4°C. The cells were centrifuged, the pellet was suspended in an equal volume of 20% bovine serum albumin (Sigma), and the mixture was polymerized with 25% glutaraldehyde. The resulting mixture was chilled on ice, cut into small blocks, and rinsed three times with 0.1 M cacodylate buffer (pH 7.0). The cells were postfixed for 1 h with 1% OsO4 in 0.1 M cacodylate buffer, and washes were repeated. Cells were dehydrated in a graded series of ethanol washes and embedded in Araldite 502 epoxy resin (J.B. EM Services Inc., Pointe-Claire, Quebec, Canada). Ultrathin sections (silver to light gold sections) were cut with an Ultracut E microtome (Reichert-Jung, Cambridge Instruments Canada Inc., Montreal, Quebec, Canada) and mounted on copper grids. Stain-

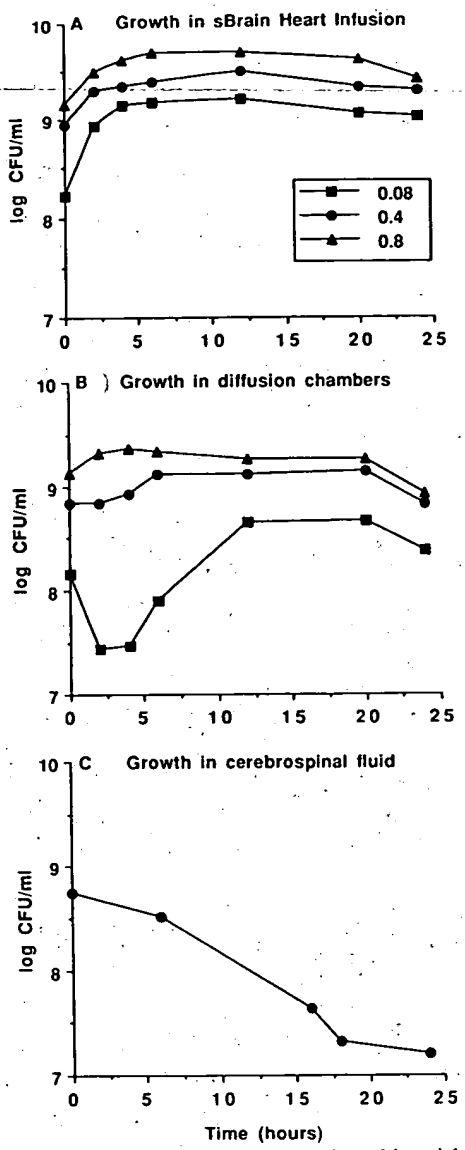

FIG. 1. Growth of *H. influenzae* monitored by viable cell plate counting at intervals in sBHI in vitro (A), after implantation of diffusion chambers in the peritoneal cavity of rats (B), and in human cerebrospinal fluids in vitro (C). The starting inocula used are indicated in A_{600} units.

ing with uranyl acetate and lead citrate was performed before examination on a Philips EM 300 electron microscope at 60 kV. In some experiments, bacterial cells were stained with ruthenium red (13).

RESULTS

Growth of *H. influenzae* in diffusion chambers and development of genetic competence. The growth of *H. influenzae* Rd was monitored after the implantation of diffusion chambers in the peritoneal cavity of rats (Fig. 1B) and was compared with the growth achieved in vitro with sBHI as the cultivation medium (Fig. 1A). *H. influenzae* cells grown in vivo were slow growing but remained viable even after 24 h of incubation in diffusion chambers implanted in rats. Figure 2 reports graphically the frequencies of genetic transformation

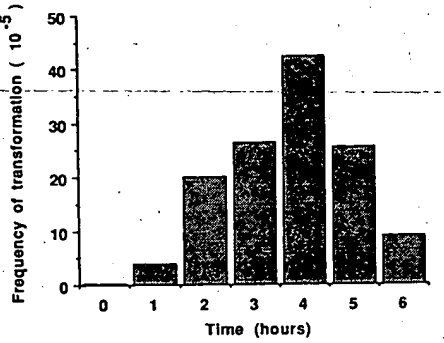

FIG. 2. Transformation frequencies obtained with bacteria grown in diffusion chambers (starting inoculum, A_{600} = 0.4). Chromosomal DNA isolated from an antibiotic-resistant donor strain was mixed with bacteria in diffusion chambers. At different times during a 5-h incubation period, recipient bacteria were collected from chambers, CFU were determined by plate counting, and antibiotic-resistant transformants were isolated on selective plates.

of cells to the antibiotic-resistant phenotype achieved when bacteria were cultivated in rats and exposed to transforming DNA (see Materials and Methods). The genetic competence of *H. influenzae* developed rapidly during growth in diffusion chambers but was lost equally rapidly after 4 h of incubation.

Binding of radiolabeled penicillin V to whole cells. The binding of radiolabeled penicillin V to target proteins (PBPs) was investigated under different growth conditions. Figure 3 shows the PBP profile of *H. influenzae* cells cultivated in rats, in which an important reduction in the binding of ^{125}I-penicillin V to PBPs 3A, 3B, 4, and 6 was observed over time. The relative reductions in the labeling of PBPs 3A, 3B, 4, and 6 after 16 h of incubation in diffusion chambers were 100, 50, 42, and 100%, respectively, as measured by densitometry. Accompanying the reduction in the labeling of the above-mentioned PBPs was a gradual appearance of a PBP of about 32 kDa, observed just above PBP 6 in gels. Typical *H. influenzae* PBP profiles were restored in cells subjected to one passage on solid medium in vitro, revealing that the PBP modifications were reversible (Fig. 3). Alterations in PBPs

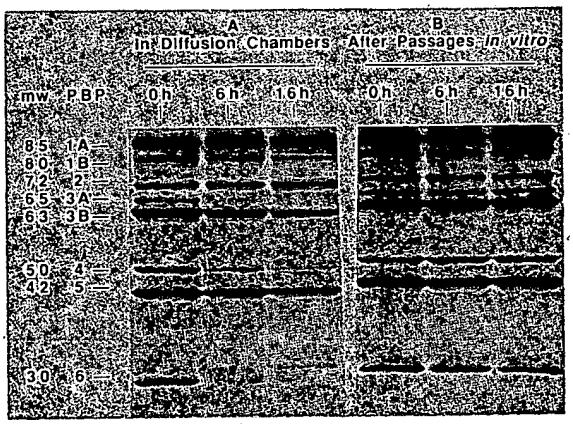

FIG. 3. Autoradiograph showing the PBP profile of *H. influenzae* cells isolated from diffusion chambers (starting inoculum, A_{600} = 0.4) (A) and after passages in vitro (B). Cells were collected and labeled with ^{125}I-penicillin V at the indicated times. The PBPs of *H. influenzae* Rd are indicated to the left (numbering system of Parr and Bryan [34]); their molecular weights (mw) are also shown (in thousands).

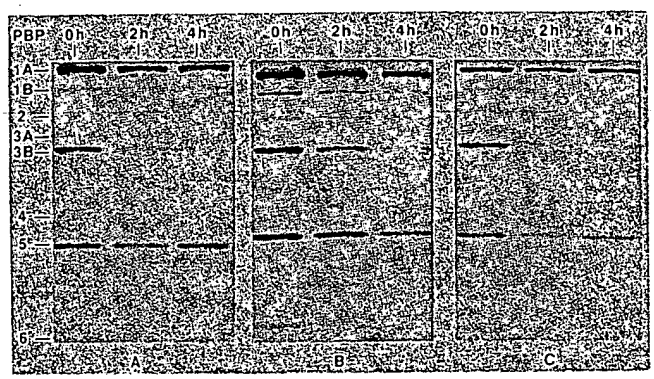

FIG. 4. Autoradiograph showing the PBP profile of *H. influenzae* cells isolated from diffusion chambers with various starting inocula. The inocula were prepared in saline and injected into diffusion chambers at an A_{600} of 0.08 (A), 0.4 (B), or 0.8 (C). Chambers were removed, and cells were collected and labeled with ^{125}I-penicillin V at the indicated times, which correspond to the datum points in Fig. 1B. The PBPs of *H. influenzae* Rd are indicated to the left.

were seen as soon as 2 h after the implantation of diffusion chambers in rats, even in experiments in which the starting inocula were small (Fig. 4).

Cells of *H. influenzae* grown in vitro in sBHI (starting inoculum, A_{600} = 0.4; 8.95 × 10^8 CFU/ml) were also labeled with ^{125}I-penicillin V. A reduction in the binding of the radiolabeled β-lactam to PBPs 3A, 3B, 4, and 6, similar to that observed with bacteria grown in diffusion chambers, was seen with cells collected during the stationary phase of growth from 20-h-old cultures. The appearance of this phenotype was much more rapid in experiments in which a larger starting inoculum was used (A_{600} = 0.8; 1.44 × 10^9 CFU/ml), as shown in Fig. 5, and again paralleled entry into the stationary phase of growth for these cells (Fig. 1A).

To reproduce in vivo growth conditions, we used the peritoneal fluid retrieved from sterile diffusion chambers as a cultivation medium for cells in test tubes. The PBP profiles of cells cultivated in this liquid showed a reduction in the binding of ^{125}I-penicillin V to PBPs 3A, 3B, 4, and 6 identical to that observed with cells grown in diffusion chambers. A pool of human cerebrospinal fluids was also used to cultivate *H. influenzae* cells in test tubes. In this case, PBP 2 (72 kDa) showed a strong reduction in binding, as did PBPs 3A, 3B, 4, and 6 (Fig. 6). The growth of *H. influenzae* in these biological

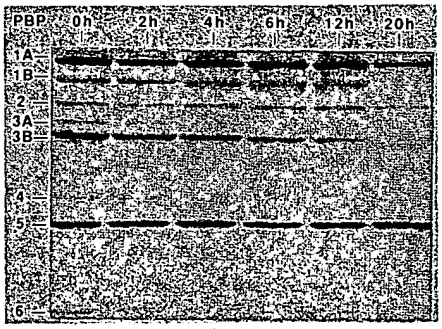

FIG. 5. Autoradiograph showing the PBP profile of *H. influenzae* cells grown in vitro in sBHI. The inoculum was used at an A_{600} of 0.8. Cells were collected and labeled with ^{125}I-penicillin V at the indicated times, which correspond to the datum points in Fig. 1A. The PBPs of *H. influenzae* Rd are indicated to the left.

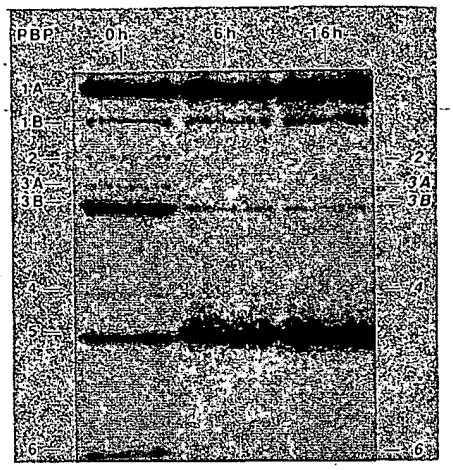

FIG. 6. Autoradiograph showing the PBP profile of *H. influenzae* cells grown in test tubes with a pool of human cerebrospinal fluids as the cultivation medium. The inoculum was used at an A_{600} of 0.4. Cells were collected and labeled with ^{125}I-penicillin V at the indicated times, which correspond to the datum points in Fig. 1C. The PBPs of *H. influenzae* Rd are indicated to the left, and the PBPs indicated on the right represent the altered proteins.

fluids was impaired, but cells remained viable (>10^7 CFU/ml) throughout the experiment (Fig. 1C). The composition of our pool of cerebrospinal fluids is given in Table 1 and was close to normal, but the pool was not artificially buffered prior to use (pH 8.9).

Other experimental in vitro growth conditions were also tested. PBPs of synchronized cells were examined. Figure 7 shows the growth of cells after an attempt at synchronization, as monitored by viable cell plate counting and measurement of the optical density of the culture. No changes in PBPs were observed during such a cell cycle (data not shown). Finally, in another set of experiments performed in vitro, we could only induce a small reduction in the binding of ^{125}I-penicillin V to PBPs by using 6-h-old sBHI cultures supplemented with an extreme amount of cAMP (100 mM) (data not shown).

Electron microscopy. Bacteria (starting inoculum, A_{600} = 0.4) were collected from diffusion chambers implanted in rats to perform electron microscopy in an attempt to correlate changes observed in the penicillin-binding properties of PBPs with possible morphological alterations of *H. influenzae* cells. As shown in Fig. 8, morphological modifications of *H. influenzae* cells occurred rapidly after implantation of

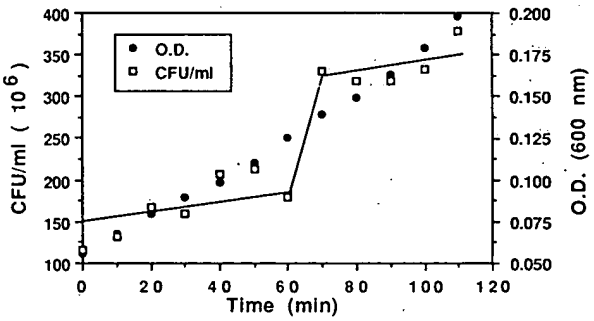

FIG. 7. Growth of cells after synchronization, as monitored by viable cell plate counting and measurement of the optical density (O.D.) of the culture.

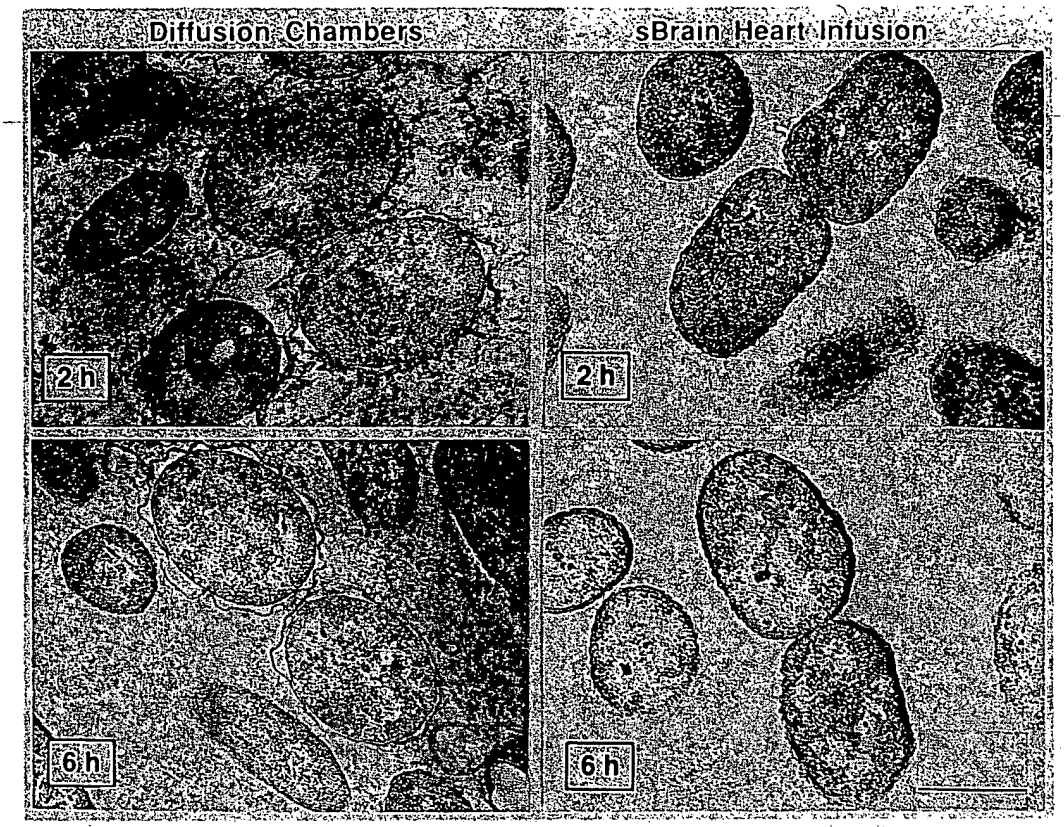

FIG. 8. Electron photomicrographs of *H. influenzae* cells cultivated in diffusion chambers and sBHI. Samples were collected and examinated at the indicated times. Bar, 0.5 μm.

diffusion chambers in rats. Pleomorphic blebs were seen on the surface of cells isolated from rats (Fig. 8) and cells cultivated in cerebrospinal fluids (data not shown) after a short incubation of 2 h. Similar cell shape irregularities were also noted in bacteria grown in vitro but only during the stationary phase (data not shown). On some cells, the blebs appeared in small multilamellar vesicles of 30 to 80 nm (Fig. 9).

DISCUSSION

Some β-lactam antibiotics are currently recommended for the treatment of *H. influenzae* infections. Although newer cephalosporins have demonstrated potent antibacterial activity in vitro and have been used successfully as therapeutic agents in severe *H. influenzae* infections, resistant or persistent bacteria may occur and cause treatment failure or delayed sterilization of biological fluids (4, 30). β-Lactam drugs exert their initial bacteriostatic effect through the inhibition of essential peptidoglycan-synthesizing enzymes, the PBPs (47). The enzymatic activities of PBPs and the overall peptidoglycan metabolism are directly affected by the physiological state of bacteria, which in turns depends on the environmental conditions. We designed this study to allow the characterization of the physiological state of cells grown in animals. The hope was to mimic in vivo growth conditions and reveal specific cell properties that may influence the inhibitory activity of β-lactams.

In the present study, we used diffusion chambers implanted in the peritoneal cavity of rats to cultivate *H. influenzae* cells. Although we recognize that this cultivation system cannot reproduce perfectly the growth conditions occurring in humans, diffusion chambers allow the recovery of a large quantity of bacteria to perform various analyses. Furthermore, we previously demonstrated the ability of this cultivation system to induce iron-regulated outer membrane proteins in *Serratia marcescens* in response to in vivo growth conditions (25). Other investigators have also successfully used diffusion chambers implanted in mice to study the efficacy of tobramycin therapy against nonmucoid and mucoid *Pseudomonas aeruginosa* in vivo (15). Here, using *H. influenzae* as the test organism in diffusion chambers, we noted important changes in cell properties. Some of the target proteins of β-lactam antibiotics (PBPs 3A, 3B, 4, and 6) showed a striking reduction in their affinity for ^{125}I-penicillin V (Fig. 3). These alterations developed as rapidly as 2 h after the implantation of diffusion chambers in rats (Fig. 4) and were associated with a peak of genetic competence (Fig. 2) and abnormal cell morphology (Fig. 8 and 9). Why the competence diminished rapidly after reaching its peak is not fully understood, but this phenomenon was also seen in vitro with M-IV medium used for the development of genetic competence (11). An additional alteration in PBP 2 was specifically seen in bacteria grown in human cerebrospinal fluids (Fig. 6). An explanation for this result was not found, except that these biological fluids were not artificially buffered to improve the growth of *H. influenzae*. The irregular and bleblike structures revealed on the surface of cells by electron microscopy (Fig. 8 and 9) may correspond to cellular structures identified during the development of genetic competence in *H. influenzae* (5), but we have no supportive evidence for this hypothesis, other than the

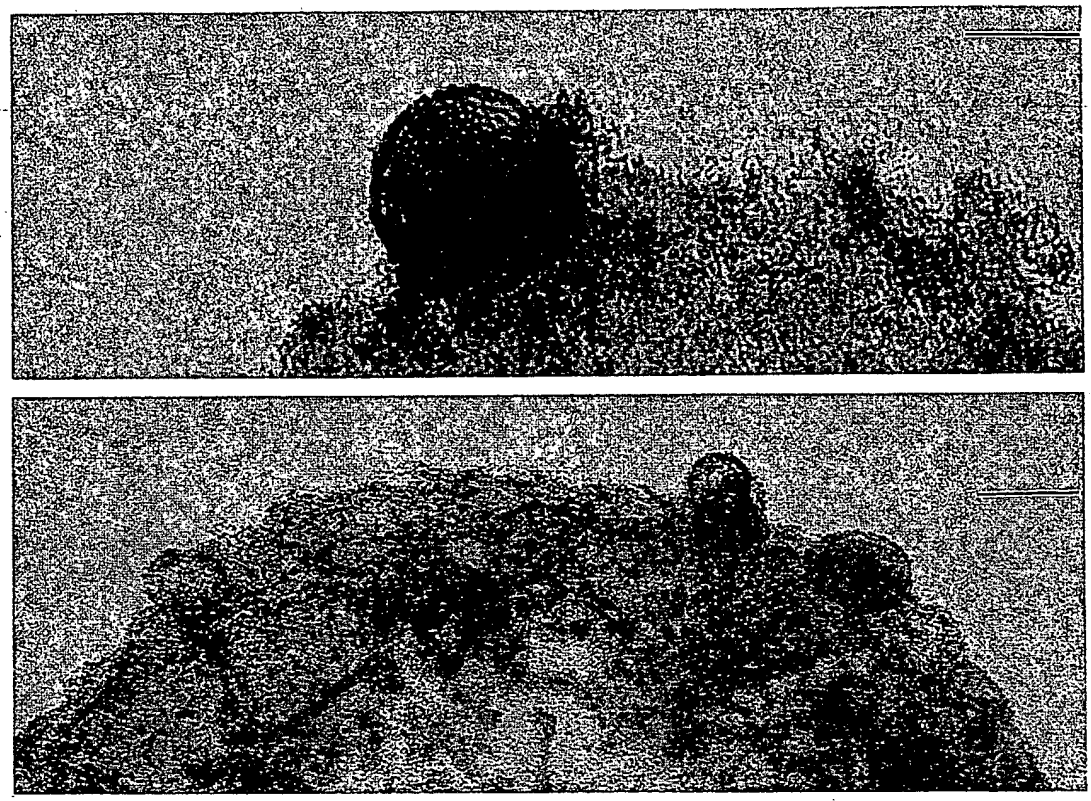

FIG. 9. Electron photomicrographs of multilamellar vesicules observed on the surface of *H. influenzae* cells. Bars, 50 nm.

concomitant high frequency of transformation observed in rats (Fig. 2). Recently, similar pleomorphic membrane blebs were observed on *Neisseria gonorrhoeae* cells, purified, and shown to be associated with plasmid DNA used as a marker in genetic transfer experiments (7). These investigators suggested that these membrane blebs or vesicles were involved in an active cellular process allowing efficient genetic exchanges between cells in vivo.

In an attempt to create in vitro a physiological state of cells similar to that observed in bacteria grown in animals or in human cerebrospinal fluids, we supplemented culture medium with cAMP. It is well recognized that cellular cAMP is responsible for the regulation of some cellular processes, such as the development of natural genetic competence in *H. influenzae* (48). In *Escherichia coli*, the initiation of cell elongation, mediated by PBP 2, has been found to be modulated by cAMP (17, 18). It has also been suggested that septation can be metabolically regulated through control of the FtsA protein by cAMP (45, 46). In addition, the β-lactam-induced lysis of bacterial cells has been shown to be cell cycle dependent in *E. coli* (8) and in staphylococci (22), and this observation implies that the activity or the expression of PBPs in bacteria may vary during a single cell cycle. We therefore also studied the PBP profiles of synchronized cells of *H. influenzae*. Although we used crude assays, neither supplemental cAMP nor attempts at cell synchronization recreated the cellular modifications and physiological state observed in bacteria grown in diffusion chambers in rats or in cerebrospinal fluids in vitro. However, a reduction in the binding affinity of PBPs 3A, 3B, 4, and 6, identical to that seen in *H. influenzae* cells cultivated in vivo, was observed in "old" broth cultures or, more precisely, in cells collected from the stationary phase of growth (Fig. 5), an observation similar to that previously reported by Mendelman and Chaffin (28).

The observation that, when exposed to an animal environment, *H. influenzae* cells rapidly enter a physiological state that is similar to that found in the late stationary phase of growth in vitro may imply the presence of an active cellular process that may have very important consequences in antibiotic therapy. A recent review outlines the importance of studying the properties of bacteria in conditions that simulate in vivo growth (21). Indeed, it is now well recognized that slow-growing *E. coli* cells are more resistant to the bactericidal action of β-lactams, a phenomenon that correlates with changes in the rate of synthesis and composition of peptidogylcan (42, 43) as well as with a decreased sensitivity of murein to autolysins (44). As one of the elements essential for an initial inhibition of cell growth by β-lactams, a slower rate of peptidoglycan synthesis by PBPs also causes resistance or tolerance to these antibiotics. In *E. coli*, the stringent response, mediated by the RelA protein, is induced by amino acid starvation. One of the effects of this response is that cells become resistant to lysis by β-lactams and other penicillin derivatives (9, 41). This RelA-dependent lysis protection is also believed to be due to a combination of both reduced peptidoglycan synthesis (12, 19) and the production of modified autolysis-resistant peptidoglycan (9, 44). Recently, Powell and Young (35) showed that the induction of heat shock proteins in *E. coli* prevented the lysis of cells by β-lactam antibiotics. They suggested that since many environmental stresses promote the expression of heat shock proteins in *E. coli*, these proteins may contribute an additional mechanism toward the development of phenotypic tolerance of β-lactams. Interestingly, we showed in previous studies that *H. influenzae* PBPs 3A, 3B, 4, and 6 were altered

by growth at 42°C (26) and that there was a correlation between the temperature sensitivity of PBPs 3A and 3B and *H. influenzae* resistance to moxalactam at 42°C (24). The effect of temperature itself could not explain the observations obtained with the diffusion chamber model, since rats maintained a rectal temperature of 34.8 ± 0.9°C throughout each experiment. However, it seems possible that an identical cellular process controls the expression of PBPs (or their activities) following an environmental stress, such as in vivo growth or heat shock.

On the basis of our findings, we speculate that during infection in humans or in healthy carriers, *H. influenzae* cells may be in a specific physiological state (similar to that demonstrated in the present study) provoked by a cellular response to an environmental stress. Such growth conditions and such a physiological state may possibly diminish antibiotic action and delay the eradication of bacteria during therapy. Our laboratory and others have shown the importance of PBPs 3A and 3B in non-β-lactamase-mediated β-lactam resistance in *H. influenzae* (3, 31, 34, 37) and the essential role of these PBPs and PBP 4 in septal peptidoglycan synthesis (26, 29). Unaltered physiological activities of PBPs 3A, 3B, 4, and perhaps 2 and 6 may be very important for efficient β-lactam action. Moreover, because of the rapid development of genetic competence in vivo, *H. influenzae* may be well disposed to facilitating the spread of antibiotic resistance. We are currently studying the inhibitory activity of β-lactam antibiotics on *H. influenzae* cells cultivated in biological fluids in the hope of identifying β-lactam drugs that will overcome the newly identified PBP alterations and that will remain active against latent or slow-growing bacteria.

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REFERENCES

1. **Beauchamp, D., M. Pellerin, P. Gourde, M. Pettigrew, and M. G. Bergeron.** 1990. Effects of daptomycin and vancomycin on tobramycin nephrotoxicity in rats. Antimicrob. Agents Chemother. **34**:139–147.
2. **Blaszczak, L. C., and N. G. Halligan.** 1988. Radioiododestannylation. Convenient synthesis of a penicillin derivative for rapid penicillin-binding protein (PBP) assay. J. Labelled Compd. Radiopharm. **27**:401–406.
3. **Clairoux, N., M. Picard, A. Brochu, N. Rousseau, P. Gourde, D. Beauchamp, T. R. Parr, Jr., M. G. Bergeron, and F. Malouin.** 1992. Molecular basis of the non-β-lactamase-mediated resistance to β-lactam antibiotics in strains of *Haemophilus influenzae* isolated in Canada. Antimicrob. Agents Chemother. **36**: 1504–1513.
4. **Dajani, A. S., and L. H. Pokowski.** 1990. Delayed cerebrospinal fluid sterilization, in vitro bactericidal activities, and side effects of selected beta-lactams. Scand. J. Infect. Dis. Suppl. **73**:31–42.
5. **Deich, R. A., and L. C. Hoyer.** 1982. Generation and release of DNA-binding vesicles by *Haemophilus influenzae* during induction and loss of competence. J. Bacteriol. **152**:855–864.
6. **Doern, G. V., J. H. Jorgensen, C. Thornsberry, D. A. Preston, T. Tubert, J. S. Redding, and L. A. Maher.** 1988. National collaborative study of the prevalence of antimicrobial resistance among clinical isolates of *Haemophilus influenzae*. Antimicrob. Agents Chemother. **32**:180–185.
7. **Dorward, D. W., C. F. Garon, and R. C. Judd.** 1989. Export and intracellular transfer of DNA via membrane blebs of *Neisseria gonorrhoeae*. J. Bacteriol. **171**:2499–2505.
8. **Garcia del Portillo, F., M. A. de Pedro, D. Joseleau-Petit, and R. D'Ari.** 1989. Lytic response of *Escherichia coli* cells to inhibitors of penicillin-binding proteins 1a and 1b as a timed event related to cell division. J. Bacteriol. **171**:4217–4221.
9. **Goodwell, W., and A. Tomasz.** 1980. Alterations of *Escherichia coli* murein during amino acid starvation. J. Bacteriol. **144**:1009–1016.
10. **Hayat, M. A.** 1986. Basic techniques for transmission electron microscopy. Academic Press Inc. (London), Ltd., London.
11. **Herriott, R. M., E. M. Meyer, and M. Vogt.** 1970. Defined nongrowth media for stage II development of competence in *Haemophilus influenzae*. J. Bacteriol. **101**:517–524.
12. **Ishiguro, E. E.** 1983. Mechanism of stringent control of peptidoglycan synthesis in *Escherichia coli*, p. 631–636. *In* R. Hakenbeck, J.-V. Holtje, and H. Labischinski (ed.), The target of penicillin. Walter de Gruyter and Co., Berlin.
13. **Jacques, M., M. Gottschalk, B. Foiry, and R. Higgins.** 1990. Ultrastructure study of surface components of *Streptococcus suis*. J. Bacteriol. **172**:2833–2838.
14. **Jorgensen, J. H., G. V. Doern, L. A. Maher, A. W. Howell, and J. S. Redding.** 1990. Antimicrobial resistance among respiratory isolates of *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae* in the United States. Antimicrob. Agents Chemother. **34**:2075–2080.
15. **Kelly, N. M., E. G. Rawling, and R. E. W. Hancock.** 1989. Determinants of the efficacy of tobramycin therapy against isogenic nonmucoid and mucoid derivatives of *Pseudomonas aeruginosa* PAO1 growing in peritoneal chambers in mice. Antimicrob. Agents Chemother. **33**:1207–1211.
16. **Klein, J. O., and S. M. Marcy.** 1983. Bacterial sepsis and meningitis, p. 679–754. *In* J. S. Remington and J. O. Klein (ed.), Infectious diseases of the fetus and newborn infant. W. B. Saunders Co., Philadelphia.
17. **Kumar, S.** 1976. Properties of *cya* and *crp* mutants of *Escherichia coli*. J. Bacteriol. **125**:545–555.
18. **Kumar, S., N. Prakash, and K. N. Agarwal.** 1979. Cyclic AMP control of the envelope growth in *Escherichia coli*: envelope morphology of the mutants in *cya* and *crp* genes. Indian J. Exp. Biol. **17**:325–327.
19. **Kusser, W., and E. E. Ishiguro.** 1986. Lysis of non-growing *Escherichia coli* by combinations of β-lactam antibiotics and inhibitors of ribosome function. Antimicrob. Agents Chemother. **29**:451–455.
20. **Laemmli, U. K., and F. Favre.** 1973. Maturation of bacteriophage T4. I. DNA packaging events. J. Mol. Biol. **80**:575–599.
21. **Lorian, V.** 1989. In vitro simulation of in vivo conditions: physical state of the culture medium. J. Clin. Microbiol. **27**: 2403–2406.
22. **Maidhof, H., L. Johannsen, H. Labischinski, and P. Giesbrecht.** 1989. Onset of penicillin-induced bacteriolysis in staphylococci is cell cycle dependent. J. Bacteriol. **171**:2252–2257.
23. **Malouin, F., and L. E. Bryan.** 1986. Modification of penicillin-binding proteins as mechanisms of β-lactam resistance. Antimicrob. Agents Chemother. **30**:1–5.
24. **Malouin, F., and L. E. Bryan.** 1988. *Haemophilus influenzae* penicillin-binding proteins 1a and 3 possess distinct and opposite temperature-modulated penicillin-binding activities. Antimicrob. Agents Chemother. **32**:498–502.
25. **Malouin, F., G. D. Cambell, M. Halpenny, G. W. Becker, and T. R. Parr, Jr.** 1990. Outer membrane and porin characteristics of *Serratia marcesens* grown in vitro and in rat intraperitoneal diffusion chambers. Infect. Immun. **58**:1247–1253.
26. **Malouin, F., T. R. Parr, Jr., and L. E. Bryan.** 1990. Identification of a group of *Haemophilus influenzae* penicillin-binding proteins that may have complementary physiological roles. Antimicrob. Agents Chemother. **34**:363–365.

27. **Malouin, F., A. B. Schryvers, and L. E. Bryan.** 1987. Cloning and expression of genes responsible for altered penicillin-binding proteins 3a and 3b in *Haemophilus influenzae*. Antimicrob. Agents Chemother. **31**:286–291.
28. **Mendelman, P. M., and D. O. Chaffin.** 1985. Two penicillin-binding proteins of *Haemophilus influenzae* are lost after cells enter stationary phase. FEMS Microbiol. Lett. **30**:399–402.
29. **Mendelman, P. M., and D. O. Chaffin.** 1987. Penicillin-binding proteins 4 and 5 of *Haemophilus influenzae* are involved in cell wall incorporation. FEMS Microbiol. Lett. **48**:239–242.
30. **Mendelman, P. M., D. O. Chaffin, L. R. Krilov, G. Kalaitzoglou, D. A. Serfass, O. Onay, E. A. Wiley, G. D. Overturf, and L. G. Rubin.** 1990. Cefuroxime treatment failure of nontypable *Haemophilus influenzae* meningitis associated with alterations of penicillin-binding proteins. J. Infect. Dis. **162**:1118–1123.
31. **Mendelman, P. M., D. O. Chaffin, T. L. Stull, C. E. Rubens, K. D. Mack, and A. L. Smith.** 1984. Characterization of non-β-lactamase-mediated ampicillin resistance in *Haemophilus influenzae*. Antimicrob. Agents Chemother. **26**:235–244.
32. **Murphy, T. F., and M. A. Apicella.** 1987. Nontypable *Haemophilus influenzae*: a review of clinical aspects, surface antigens, and the human immune response to infection. Rev. Infect. Dis. **9**:1–15.
33. **Nikaido, H.** 1989. Role of the outer membrane of gram-negative bacteria in antimicrobial resistance, p. 1–34. *In* L. E. Bryan (ed.), Microbial resistance to drugs. Springer-Verlag, Berlin.
34. **Parr, T. R., Jr., and L. E. Bryan.** 1984. Mechanism of resistance of an ampicillin-resistant, β-lactamase-negative clinical isolate of *Haemophilus influenzae* type b to β-lactam antibiotics. Antimicrob. Agents Chemother. **25**:747–753.
35. **Powell, J. K., and K. D. Young.** 1991. Lysis of *Escherichia coli* by β-lactams which bind penicillin-binding proteins 1a and 1b: inhibition by heat shock proteins. J. Bacteriol. **173**:4021–4026.
36. **Preston, D. A., C. Y. E. Wu, L. C. Blaszczak, D. E. Seltz, and N. G. Halligan.** 1990. Biological characterization of a new radioactive labeling reagent for bacterial penicillin-binding proteins. Antimicrob. Agents Chemother. **34**:718–721.
37. **Serfass, D. A., P. M. Mendelman, D. O. Chaffin, and C. A. Needham.** 1986. Ampicillin resistance and penicillin-binding proteins of *Haemophilus influenzae*. J. Gen. Microbiol. **132**: 2855–2861.
38. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions, p. 137–139. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Spratt, B. G.** 1977. Properties of the penicillin-binding proteins of *E. coli* K-12. Eur. J. Biochem. **72**:341–352.
40. **Stewart, G. J., and C. A. Carlson.** 1986. The biology of natural transformation. Annu. Rev. Microbiol. **40**:211–235.
41. **Tuomanen, E.** 1986. Phenotypic tolerance: the search for β-lactam antibiotics that kill non-growing bacteria. Rev. Infect. Dis. **8**(Suppl. 3):S279–S291.
42. **Tuomanen, E., and R. Cozen.** 1987. Changes in peptidoglycan composition and penicillin-binding proteins in slowly growing *Escherichia coli*. J. Bacteriol. **169**:5308–5310.
43. **Tuomanen, E., D. T. Durack, and A. Tomasz.** 1986. Antibiotic tolerance among clinical isolates of bacteria. Antimicrob. Agents Chemother. **30**:521–527.
44. **Tuomanen, E., Z. Markiewicz, and A. Tomasz.** 1988. Autolysis-resistant peptidoglycan of anomalous composition in amino-acid-starved *Escherichia coli*. J. Bacteriol. **170**:1373–1376.
45. **Utsumi, R., Y. Nakamoto, M. Kawamukai, M. Himeno, and T. Komano.** 1982. Involvement of cyclic AMP and its receptor protein in filamentation of an *Escherichia coli fic* mutant. J. Bacteriol. **151**:807–812.
46. **Utsumi, R., H. Tanabe, Y. Nakamoto, M. Kawamukai, H. Sakai, M. Himeno, T. Komano, and Y. Hirota.** 1981. Inhibitory effect of adenosine 3′,5′-phosphate on cell division of *Escherichia coli* K-12 mutant derivatives. J. Bacteriol. **147**:1105–1109.
47. **Waxman, D. J., and J. L. Strominger.** 1983. Penicillin-binding proteins and the mechanism of action of β-lactam antibiotics. Annu. Rev. Biochem. **52**:825–869.
48. **Wise, E. M., S. P. Alexander, and M. Powers.** 1973. Adenosine 3′:5′-cyclic monophosphate as a regulator of bacterial transformation. Proc. Natl. Acad. Sci. USA **70**:471–474.
49. **Zhanel, G. G., J. A. Karlowsky, R. J. Davidson, and D. J. Hoban.** 1992. Effect of pooled human cerebrospinal fluid on the postantibiotic effects of cefotaxime, ciprofloxacin, and gentamicin against *Escherichia coli*. Antimicrob. Agents Chemother. **36**:1136–1139.